ENVIRONMENTALLY CONTROLLED INDUCTION OF PRIMARY MALE GONOCHORISTS FROM EGGS OF THE SELF-FERTILIZING HERMAPHRODITIC FISH, RIVULUS MARMORATUS POEY

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The fact of genetic sex determination among teleosts is well established by sex linkage (Gordon, 1957), although cytological demonstrations of fish sex chromosomes have not withstood critical scrutiny (White, 1954) until recently (Nogusa, 1960). Both male and female fish with phenotypic sex contrary to genotypic sex have been produced by early treatment with sex steroids (Yamamoto, 1953–1961). Can genetic sex determination in fishes be overridden also by external environmental factors, as in some amphibians, is the question to which answers were sought in the experiments to be reported here.

This question was first raised by effects on anuran sex determination of delayed fertilization (overripeness of eggs) and of temperature obtained, respectively, by Pflüger (1882) and Witschi (1929). Comparable experiments on fishes have been few; their long duration with no assurance of negotiable results discourages investigation. Under harsh contrasting experimental conditions it is extremely difficult to rear fish through the early crises of ontogeny without excessive losses, and if mortalities exceed a certain limit, the dilemma of a differential mortality of one sex *versus* experimental induction of the other cannot be resolved.

Conclusive evidence of environmental influence on sex determination in teleosts is lacking despite possible indications of such influence from experiments on one species each of the genera *Salmo* (Mršić, 1923), *Betta* (Eberhardt, 1943), and *Anguilla* (D'Ancona, 1950, 1960). Only by making explicit certain crucial defects in these experiments passed over by reviewers can the rationale of our own experiments and the cogency and singularity of their results be given their full context (see Discussion).

The cyprinodontid fish used in the present study, *Rivulus marmoratus* Poey, is unique among fishes so far as known in being comprised of natural, consistently self-fertilizing hermaphrodites (Harrington, 1961, 1963; comments of Atz, 1964). Its hermaphroditism is normal and not a laboratory artifact like that of *Lebistes reticulatus* (Spurway, 1957), for example. Long deemed merely a nominal species (Garman, 1895), *R. marmoratus* was revived as a valid species by Rivas (1945), who rediscovered its types in the U. S. National Museum, but was unknown as a living fish until it was found in Florida (Harrington and Rivas, 1958). Tissue grafts between Florida wild-caught progenitors and their laboratory-reared descendants gave the *autograft reaction* (Kallman and Harrington, 1964), indicating that they have the same genotype and that probably their wild antecedents also had reproduced by self-fertilization, *i.e.* for upwards of 10 generations (see below).

Although an allegedly gonochoristic subspecies, *R. marmoratus bonairensis,* was described from the Antilles (Hoedeman, 1958) the same year that we found *R. marmoratus* in Florida, we have no evidence from the laboratory or from the wild that females exist in Florida and so far have found no males in the wild. It came as a surprise, therefore, when males appeared among hermaphrodites propagated in our laboratory, especially since these were bright orange with the caudal ocellus obsolescent, in sharp contrast to the hermaphrodites. The incidence of males has

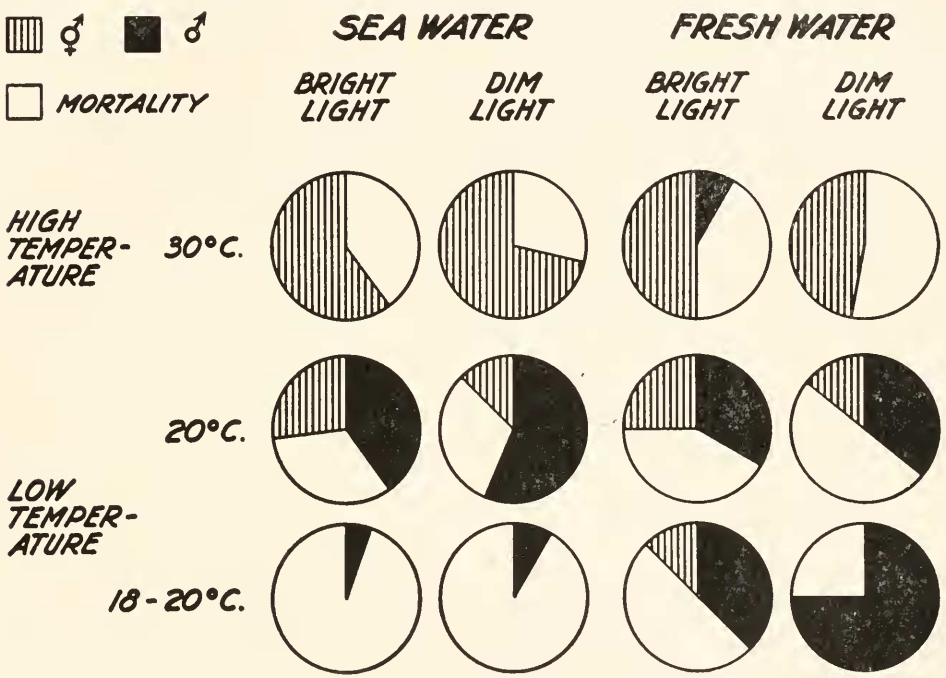

FIGURE 1. Plan and results of Experimental Series One, data in Table I. Individuals of *Rivulus marmoratus,* each in its own jar, were exposed to the eight combinations of bright or dim light, sea water or fresh water, high or low temperature. Exposure was from not later than the ¾ blastoderm stage until sexual maturity at high temperature or five months post-hatching at low. Circles show the percentages of hermaphrodites and males and the percentage mortality resulting from exposure to each extrinsic factor combination. The temperature of the lowermost row of treatments was raised to 20° C. when 18° C. proved lethal in combination with sea water, and the middle row, at 20° C., was added. Compare with the results of Experimental Series Two (Table IV).

stayed below 5% through more than 10 uniparental laboratory generations, numbering over 350 fish, each isolated throughout life to exclude physiological interactions of any kind except visual ones.

The appearance of an occasional male in clones otherwise composed of hermaphrodites suggests some lability in the sex-determining mechanism through which the genotype normally produces the hermaphrodite phenotype. It seems proper to speak here of a hermaphrodite genotype, because as Atz (1964) observes, the assumption is false that *normal hermaphroditism* cannot be genetically controlled,

as is the sex of gonochorists. The present experiments were contrived to identify a possible external environmental factor capable of causing a deviation to the male phenotype during sex differentiation. Positive results were obtained in two series of experiments, the first begun in August, 1961, the last completed in January, 1965.

Materials and Methods

Few if any experiments on vertebrates can have used material as genetically uniform as the *Rivulus marmoratus* eggs used here. In Experimental Series One

Table I

Effects of external factors on the sex ratio of uniparental offspring of Rivulus marmoratus *hermaphrodites. Self-fertilized eggs were reared from outset of extraparental incubation under various combinations of light intensity, salinity and temperature.*
B, bright light; D, dim light; S, sea water; F, fresh water;
18/20°C., started at 18° C. but continued at 20° C.

Treatment		Surviving/ treated	Percentage survival	Survivors				Non-survivors					
				Hermaphrodites		Males		Died *in ovo* or at hatching		Died very small		Extremely abnormal; discarded	
				Total	%	Total	%	Total	%	Total	%	Total	%
30° C.	B S	6/10	60.0	6	100.0		0.0	4	100.0		0.0		0.0
	D S	5/7	71.4	5	100.0		0.0	1	50.0	1	50.0		0.0
	B F	7/12	58.3	6	85.7	1	14.3	2	40.0	3	60.0		0.0
	D F	8/17	47.1	8	100.0		0.0	6	66.7	1	11.1	2	22.2
20° C.	B S	10/15	66.7	4	40.0	6	60.0	4	80.0		0.0	1	20.0
	D S	11/16	68.8	2	18.2	9	81.8	3	60.0		0.0	2	40.0
	B F	7/12	58.3	3	42.9	4	57.1	1	20.0	2	40.0	2	40.0
	D F	7/14	50.0	2	28.6	5	71.4	2	28.6	5	74.1		0.0
18/20° C.	B S	1/19	5.3		0.0	1	100.0	16	88.9		0.0	2	11.1
	D S	1/12	8.3		0.0	1	100.0	5	45.4	3	27.3	3	27.3
	B F	4/8	50.0	1	25.0	3	75.0	1	25.0	1	25.0	2	50.0
	D F	6/8	75.0		0.0	6	100.0		0.0	2	100.0		0.0
30° C.	All	26/46	56.5	25	96.2	1	3.8	13	65.0	5	25.0	2	10.0
20° C.	All	35/57	61.4	11	31.4	24	68.6	10	45.5	7	31.8	5	22.7
18/20° C.	All	12/47	25.5	1	8.3	11	91.7	22	62.9	6	17.1	7	20.0

(Fig. 1 and Table I), the fish surviving to be sexed hatched from eggs of hermaphrodites of two clones, 32 Clone-NA eggs and 41 Clone-DS eggs (Table II). The fish of Table II are coded as they were when used in the graft tests providing the evidence for these clones (Kallman and Harrington, 1964). Contrary to two data (*loc. cit.*, Table III, #II and #IV), however, Fish DS, Fish FT, and Fish NSU all belong to the same clone, later interline grafts (unpublished) having given the autograft reaction, showing that the previous rejections (#II and #IV) were mechanical and not immunological. In Experimental Series Two (Table IV), all were Clone-DS eggs of Uniparental Laboratory Generations 9, 10, and 11, so that

TABLE II

Sex ratios of progeny of self-fertilized Rivulus marmoratus *reared* ab ovo *from outset of extraparental incubation at either high or low temperature, showing the same temperature correlation regardless of parentage or clone.* Same data as in Table I*

Parent	Progeny surviving/ treated	Percentage survival	High temperature (30° C.)				Low temperature (18–20° C.)			
			Hermaphrodites		Males		Hermaphrodites		Males	
			Total	%	Total	%	Total	%	Total	%
FT	19/42	45.2	5	100.0		0.0	2	14.3	12	85.7
NSU	2/2	100.0	1	100.0		0.0		0.0	1	100.0
DS P_1	6/11	54.5	3	100.0		0.0	1	33.3	2	66.7
F_1	6/28	21.4	1	100.0		0.0	3	60.0	2	40.0
F_2	8/14	57.1	1	100.0		0.0	4	57.1	3	42.9
NA	32/52	61.5	14	93.3	1	6.7	2	11.8	15	88.2
NSB	0/1	0.0								
Totals	73/150	48.7	25	92.6	1	3.8	12	25.5	35	74.5

* Wild-caught fish FT, NSU, and DS and their uniparental descendants belong to the same clone; wild-caught NA belongs to a different clone (Kallman and Harrington, 1964, and unpublished).

TABLE III

Sex ratios of progeny of self-fertilized Rivulus marmoratus *reared at either high or low temperature, showing the same temperature correlation regardless of developmental stage at outset of treatment (= outset of extraparental incubation). Same data as in Tables I–II.* Stage 1 is the fertilized egg before polar cap formation; at Stage 13c the blastoderm encloses $\frac{3}{4}$ of the yolk. For intervening stages see Harrington, 1963

Developmental stage at outset	Surviving/ treated	Percentage survival	High temperature (30° C.)				Low temperature (19–20° C.)			
			Hermaphrodites		Males		Hermaphrodites		Males	
			Total	%	Total	%	Total	%	Total	%
1	0/1	0.0								
2	1/4	25.0						0.0	1	100.0
3	1/5	20.0						0.0	1	100.0
4	5/13	38.5	4	80.0	1	20.0				
5	8/16	50.0	3	100.0		0.0	2	40.0	3	60.0
6	16/21	76.2	4	100.0		0.0	1	8.2	11	91.8
7	5/15	33.3	2	100.0		0.0	1	33.3	2	67.0
8a	4/6	66.7	2	100.0		0.0		0.0	2	100.0
8b	5/10	50.0	2	100.0		0.0		0.0	3	100.0
8c	2/6	33.3					2	100.0		0.0
8d	9/16	56.3	2	100.0		0.0	2	28.6	5	71.4
9	5/17	29.4	1	100.0		0.0	3	75.0	1	25.0
10	6/9	66.7	2	100.0		0.0	1	25.0	3	75.0
11	0/1	0.0								
12b	3/4	75.0	2	100.0		0.0		0.0	1	100.0
13a	1/2	50.0	1	100.0		0.0				
13c	2/3	66.7						0.0	2	100.0
Totals	73/150	48.7	25	96.2	1	3.8	12	25.5	35	74.5

besides the immunological evidence that the fish at the outset of these generations were of one clone (Kallman and Harrington, 1964), selfing through eight generations alone would have brought them to over 99% homozygosity (Sinnott and Dunn, 1939; p. 284). Alternatively, in the remote contingency of a homozygote-preventing mechanism, they would share the same heterozygous genotype.

The wild-caught progenitors of Table II were isolated from date of capture. Every other fish referred to in this report was kept in lifelong isolation begun at its retrieval as a self-fertilized egg being emitted by its parent. Eggs of *R. marmoratus* are laid after *intraparental incubation* for from a few minutes to 2½ days, *viz.* from in Stage 1 (just fertilized) to in Stage 24 (prominent pectoral fin buds), as before described (Harrington, 1963). Eggs for our experiments were sucked into a pipette as they fell from laying hermaphrodites, kept at a water temperature of about

TABLE IV

The sex determination and differentiation of uniparental Rivulus marmoratus *modified by temperature. Self-fertilized eggs from hermaphrodites of a single clone were reared under contrasting temperature regimes, but with light intensity and salinity controlled. Compare with Table I and Figure 1*

Temperature regime	Total eggs reared	Hermaphrodites		Males		Mortality	
		No.	%	No.	%	No.	%
A) 25 ± 1° C. to maturity (control)	50	50	100	0	0	0	0
B) 25 ± 1° C. through hatching; 19.5 ± 0.5° C. for the first 5 months post-hatching; 25 ± 1° C. thereafter to maturity	50	46	92	0	0	4	8
C) 25 ± 1° C. to at least Stage 16 but not beyond Stage 22a; then 19.5 ± 0.5° C. through eclosion* and for 5 months post-eclosion; thereafter 25 ± 1° C. to maturity	50	9	18	36	72	5	10

* Eclosion refers to either hatching or being cut out of the chorion.

25° C. Developmental stages at outset of experimental treatment varied according to the experiment. Each egg of suitable stage was pipetted into its own jar. The egg in its own jar was put under the conditions of its allotted treatment, encompassing *extraparental incubation,* hatching and subsequent life in this jar, except for one low-temperature treatment (Table IV, B) begun with hatchlings.

Wide-mouthed, straight-sided, cylindrical, screw-top jars were used. These were about 15 cm. high and 8 cm. in diameter, holding 950 ml., and were filled with 600 ml. of water. Plastic Petri-dish covers used as lids prevented escape, without injury to jumping fish or interference with gas exchange. Fish have lived in these jars for 45 months, and as many as 500 were kept concurrently, each in its own jar, during our experiments. Unremitting care was taken to exclude any possibility of transfer between jars of physiological substances. A utensil inserted in one jar was rinsed repeatedly in a container overflowing with fast-running water before being inserted in another.

Jars going from propagating room (25° C.) to the low experimental temperature (constant-temperature room) were moved immersed in a water bath at 25° C. When the jar water reached the low temperature, the jars were taken out, wiped off, and left in the constant-temperature room. Jars going to the high experimental temperature were put in a water bath to raise the jar water to this high temperature. Then the jars were moved to the constant-temperature room and left immersed for the duration of the treatment in water baths thermostatically controlled to maintain the high experimental temperature. These procedures were reversed when fish were returned to the propagating room for post-treatment observation. The conditions of each experimental treatment were maintained continuously. As new eggs became available in temporal succession, each was allocated to one of the treatments singly and in its own jar. Each jar was removed from the treatment conditions and returned to the propagating room when its fish reached functional sexual maturity, or after five months in the case of low-temperature treatments.

The fish in jars returned to the propagating room for post-treatment observation were kept in 40% sea water for the remainder of their lifelong isolation. If not originally in 40% sea water, they were changed to it gradually over three days from their former (treatment) salinity. The propagating room received light within the natural daily photoperiod only, and the room temperature was constrained by an air conditioner and thermostatically controlled heater to hold the water temperature to about 25° C.

With the release of the hatchling from its egg *chorion* (terminology of Lord Rothschild, 1958), feeding was begun, first with microworms (nematodes), then with these mixed with brine shrimp (*Artemia*) nauplii. A premature diet of *Artemia* can cause death through intestinal stoppage, the shift to food of larger size evidently being a crisis of ontogeny. Afterwards, brine shrimp alone were used. Feeding was *ad libitum;* the amount squirted into the jar with a syringe was adjusted to fish size and to volume of unconsumed food in its jar each morning. Unless otherwise stated, food was introduced in water of the same salinity as in the jar. After feeding began, the jar water was filtered weekly, by pouring through filter paper on a glass funnel into a clean jar. The fish was transferred by syringe or net to the filtrate when it was deep enough. The old jar was washed, and filtrate and fish poured back into it. The new jar, the funnel, and the syringe or net were washed, and, with fresh filter paper, used for the next filtering. Cloudy water was replaced. New water replacing old or added to compensate for loss was of the same temperature and salinity. Jar water was kept a pale blue with methylene blue. Without this bacteriostatic dye eggs and larvae seemed to have a lower survival, but this was not tested experimentally. One or more times a day, solid wastes, uneaten food, and later on, eggs, if laid by the then mature fish, were sucked out with a syringe.

The jars were monitored daily, at first for hatchlings or eggs in Stage 31, which precedes hatching (Stage 32) under natural conditions, and for abnormal, sick and dead eggs or hatchlings, later for the first external signs of sex differentiation. Throughout the life of each hermaphrodite a daily record was kept of the number, conditions, and stages of eggs found in its jar. Under certain treatments, environmental cues normally triggering the hatching mechanism were either absent or nullified (*cf.* Kinne and Kinne, 1962), because hatching proved to be another crisis

of ontogeny. Prolonged delays caused deaths through inanition (but see Harrington, 1959). In the first series of experiments, the mechanism could sometimes be activated by focusing bright light on overdue eggs, but it became the practice to cut from their chorions other embryos of the same age as those hatching naturally or with light stimulation (Table V). In the second series, the crisis was circumvented by cutting out all embryos incubated at low temperature (Table IV, C) long before the normal hatching stage, allowing the rest (Table IV, A and B) to hatch naturally, and mortalities did not exceed statistically permissible limits in the second series as they did in the first.

Hermaphrodites and males at the same temperature become externally recognizable as such at about the same age and size. Hermaphrodites retain the caudal ocellus possessed by both juvenile hermaphrodites and juvenile males, and acquire no orange pigmentation later. Sperm production in hermaphrodites is not copious enough to be visible as milt. The characteristic behavior pattern leading up to oviposition (Harrington, 1961) is confined to hermaphrodites. Each hermaphrodite was performance-proven in lifelong isolation by laying eggs from which normal fish hatched. Maturing males first acquire scattered, small orange spots on the body and minute orange flecks in increasing density on the fins. An orange wash later covers the whole body, as the caudal ocellus becomes obsolescent or vanishes altogether. Handled males often release milt, which in hanging-drop suspension shows active spermatozoa.

It is of fundamental importance to make clear that these are males *ab initio,* sometimes called "pure males," but more exactly, *primary male gonochorists.* This was established by serial sections of over 200 gonads from ontogenetic series of both hermaphrodites and males, ranging from germ cell entry into genital ridges to senility, as well as by contrast with testes of *secondary male gonochorists,* that may arise from older hermaphrodites by involution of the ovarian component of the ovotestes with proliferation of the testicular component, under conditions to be described in a separate report. Embryological and histological details are beyond the scope of the present report, but will be given elsewhere. A testis and an ovotestis, each in transverse section, and a male and hermaphrodite, both adults, are shown in Figure 2.

Experimental Series One

The treatments were the eight combinations of high *versus* low temperature, sea water *versus* fresh water, and bright *versus* dim light (Fig. 1 and Table I). Eggs ranged from the one-cell stage to Stage 13c ($\frac{3}{4}$ blastoderm) at outset of treatment (Table III), which began within five minutes after the egg was emitted by its parent. The constant-temperature room thermostat was set at first to give a low water temperature of 18° C., but this proved lethal in combination with sea water (Fig. 1 and Table I). Survivors begun at 18° C. were continued at 20° C., and eggs of a new set were started at 20° C. These low water temperatures actually fluctuated between 20° C. and 21° C., being mostly closer to 20° C., and will be referred to as 20° C. for convenience. The high-temperature jars were immersed to the level of the water within them. They rested on a wire-screen platform above the bottom, their lids just clearing the underside of the glass aquarium covers. The bath water was circulated by air stones to equalize the temperature, held by thermo-

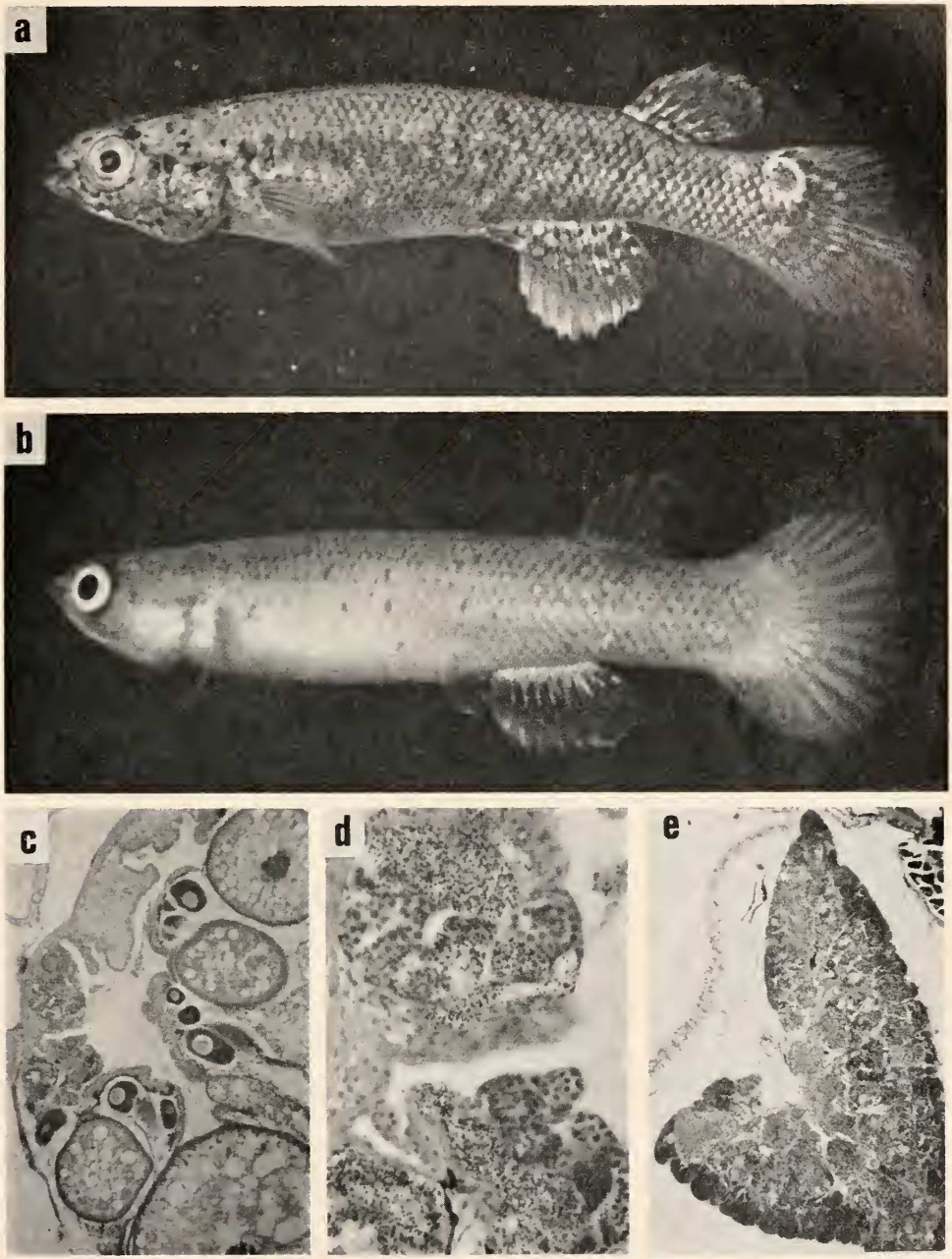

FIGURE 2. Young adult *Rivulus marmoratus*. a, hermaphrodite; b, primary male gonochorist; c, cross section of right lobe of ovotestis; d, its testicular component at higher magnification; e, cross section of right lobe of the testis of a primary male gonochorist, same magnification as in c.

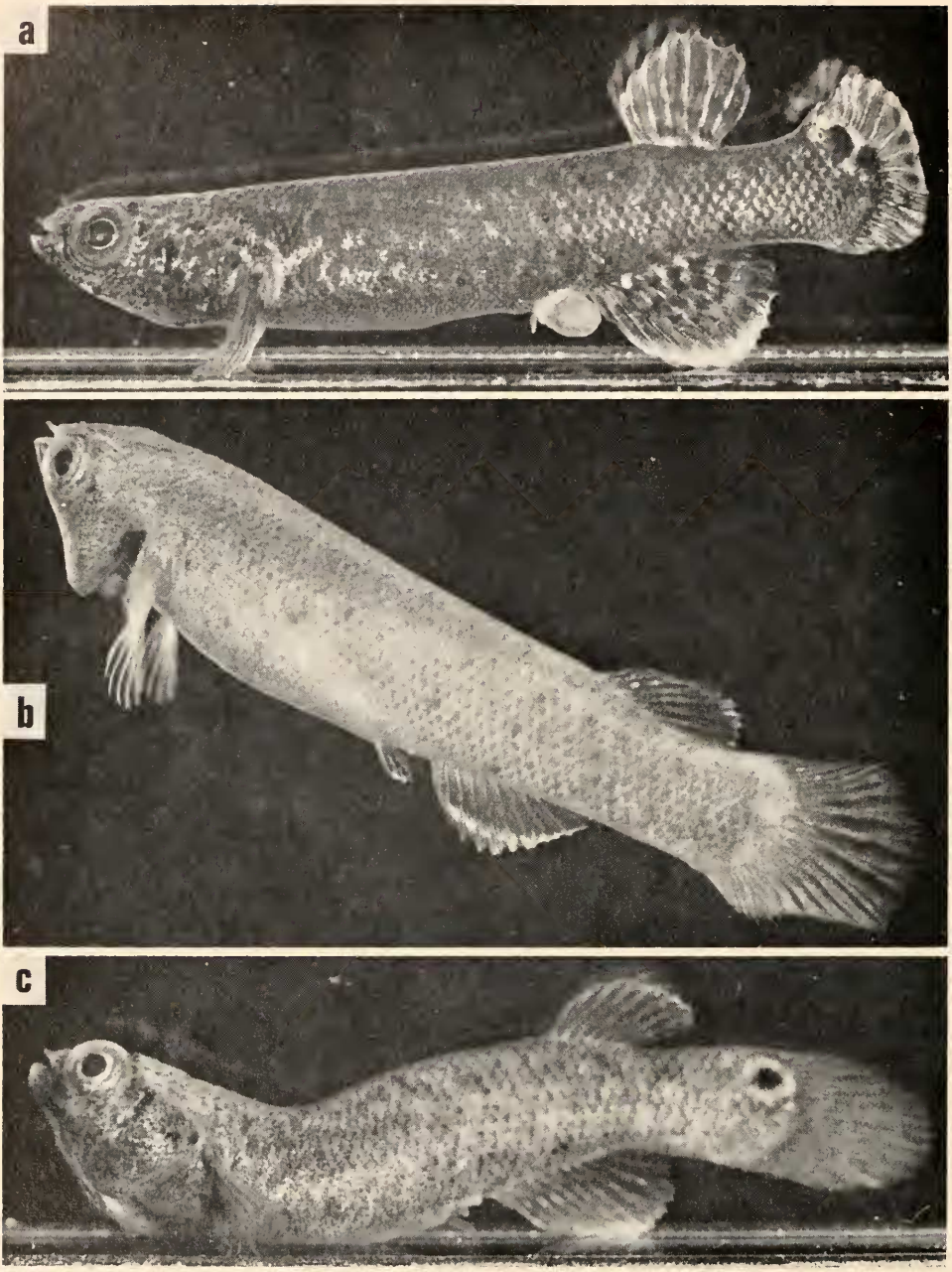

FIGURE 3. Structural-functional abnormalities of *Rivulus marmoratus* exposed to certain light-salinity-temperature combinations. Compare with Figure 1. a, *prolapsed oviduct;* confined to the dim-light, sea-water, high-temperature treatment; in 100% of the survivors; b, *pharyngeal hyperplasia;* confined to the dim-light, fresh-water, low-temperature treatments, whether begun at 18° C. and continued at 20° C., or at 20° C. throughout; in 100% of the

stat within 30 ± 1° C. Jars contained either filtered 100% sea water (salinity, 36‰) or bottled drinking water ("Blue Crystal"). Illumination was provided by four 40-watt Westinghouse Daylight fluorescent lamps suspended from above and controlled by a time switch giving a 14-hour photoperiod (0500h–1900h). Bright-lit jars received direct light of low daylight intensity (425–520 lux). Dim-lit jars were within a cube-shaped enclosure covered with black cloth on all but one side, either in a water bath or on shelves. They received indirect light of low intensity (2.55–21.00 lux), *i.e.*, mostly above the lower end of the intensity range (3.5–400.0 lux) of civil twilight (see Nielsen, 1961, 1963). Before hatching occurred, jars were examined once a day by flashlight; afterwards, although the hatchlings could feed by sight, each jar was taken out into the direct light for less than a minute each day to be checked and cleaned.

Results of Experimental Series One

Of the 150 eggs allotted to the eight treatments, 73 or 40.7% survived to sexual differentiation and functional maturity. Thirty-seven or 50.7% of these were hermaphrodites; 36 or 49.3% were males, an absolute number of males over seven times the total number encountered before the experiments were performed. All but one experimental male were from the low-temperature treatments (Table I). Figure 1 shows the incidence of males and hermaphrodites and the mortalities as percentages of the total number of eggs per treatment. In Table I, the data are given in actual numbers and also as percentages of survivors and non-survivors.

There is no indication that the incidence of males *versus* that of hermaphrodites was affected by the alternative salinities and light intensities in any of their four possible combinations, even though two light-salinity-temperature combinations resulted in far higher mortalities than the rest, *viz.*, either bright or dim light with sea water at 18° C. (see above). This is obvious from Figure 1, and warrants placing all high-temperature fish in one group and all low-temperature fish in another, as is done variously in Tables I–III. The correlation of male incidence with low-temperature rearing holds regardless of parentage or clone (Table II), or of embryonic stage at outset of treatment between Stages 1 and 13c (Table III).

There are indications, besides, that death was caused differently among the various light-salinity-temperature treatments, making it unlikely that the alternative temperatures *per se* caused alternative differential mortalities of hermaphrodites *versus* males. These indications are structural-functional abnormalities peculiar to certain light-salinity-temperature treatments. The names given them here, *prolapsed oviduct*, *pharyngeal hyperplasia*, and *kyphosis*, are intended to be no more than descriptive (Fig. 3). These abnormalities suggest that some deaths came from extreme expression of the abnormality or stress peculiar to the light-salinity-temperature treatment concerned.

Prolapsed oviduct was confined to the dim-light, sea-water, high-temperature treatment, and appeared in 100% of the survivors. It may be defined as oviposition into a non-patent, exserted oviduct. The oviduct protrudes from the genital

survivors; c, *kyphosis;* of less than 100% incidence in the fish of two complementary treatments both with dim light, one with fresh water and high temperature, the other with sea water and low (18° C. changed to 20° C., and 20° C. throughout); often accompanied by non-buoyancy and thinness of body; commoner in the low-temperature treatments.

pore as a flaccid, blind sac, filled with expelled eggs that ultimately break down. The tip of one sac was snipped off, and subsequent ovipositions were successful. The abnormality finally corrected itself in the other fish, and each in the end produced viable hatchlings, as in all the other treatments. Prolapsed oviduct occurs infrequently among *R. marmoratus* routinely propagated, and occasionally is fatal.

Pharyngeal hyperplasia was confined to the dim-light, fresh-water, low-temperature treatments, whether begun at 18° C., and continued at 20° C. or at 20° C. throughout. It occurred in 100% of the survivors of these treatments, male or hermaphrodite. It shows externally as permanently raised opercula, gaping widely and exposing basibranchial swellings. One fish head was sectioned and found to have profuse thyroid tissue, some of it apparently usurping branchial cartilage, so that the condition may tentatively be diagnosed as thyroid hyperplasia, with the reservation that no control material was sectioned.

Kyphosis (Rasquin and Rosenbloom, 1954) shows best in roentgenogram or after clearing and staining with alizarin, but was intense enough here to show up externally, although mild cases may have eluded recognition. Unlike the other two abnormalities, it fell short of 100% occurrence and was often associated with non-buoyancy and thinness of body. Kyphosis occurred in fish of two complementary treatments both with dim light, one with fresh water and high temperature, the other with sea water and low temperature (18° C. changed to 20° C. and 20° C. throughout). It was more prevalent in the low-temperature treatments and accompanied several early deaths.

Apart from these plausible causes of death, a large portion of the fish died from failure to hatch (Table I), and some deaths were probably the delayed results of weakness caused by abnormally prolonged deferment of hatching.

Post-Treatment Observations

Eggs entered treatments August 9–November 15, 1961; treatments ended January 9–May 16, 1962. Post-treatment monitoring extended to June 1, 1965, when the last survivors were fixed for sectioning. Males and hermaphrodites died or were killed each year of the post-treatment observation period, 1962–1965. Males remained unchanged except for senile degeneration, but each year some hermaphrodites transformed to secondary male gonochorists (see Material and Methods). All fish were autopsied. Eight primary males, all of the secondary males, and all but one hermaphrodite were serially sectioned. Testes of secondary males are larger than those of primary males and apparently yield more spermatozoa.

Nearly 60% of the 37 original hermaphrodites had become secondary males by the time the last fish were killed at the end of May, 1965. Possibly some that died earlier and some killed at the end of the post-treatment observation period might also have changed over had they lived longer. Of the 16 fish dying as hermaphrodites, eight died natural deaths, three of them egg-bound; of those killed, five were egg-bound and probably doomed although otherwise healthy when killed, while the remaining three were healthy when killed at the end of May, 1965.

The original 37 hermaphrodites, including those later transforming into secondary males, were kept alive, isolated and under daily observation, for 262–1376 (average, 1041) days post-hatching; the original 36 males, for 327–1314 (average, 945) days. The days from first to last egg laid by hermaphrodites dying as such

were 46–1117 (average, 694), by hermaphrodites later changing to secondary males, 107–1167 (average, 711). Secondary males acquire orange pigmentation like that of primary males, sometimes lose the caudal ocellus, which otherwise becomes obsolescent, but retain the basic hermaphrodite color pattern not shared with primary males, and are easily distinguished from primary males. The male attributes appear gradually, making their earliest recognition variable, so that the change from functional hermaphrodite to functional secondary male is best dated retrospectively, as the day on which the last egg was evacuated. Secondary males were kept alive beyond the day on which the last egg was laid (last day as hermaphrodite) for 48–918 (average, 346) days. No secondary male gonochorist reverted to its former (hermaphrodite) state. The serial sections provided detailed evidence of the transformation of ovotestes into testes. There was no histological evidence of change in the opposite direction.

The modalities of this sex inversion, from hermaphrodite to secondary male gonochorist, will be analyzed more closely in a separate report. Primary sex determination and differentiation are the concerns of the present report. These are distinct from the phenomenon of sex inversion, and with one exception this sex inversion took place after a life as a functional hermaphrodite as long as might be expected to be the life span in the wild of a fish species of this small size.

Experimental Series Two

Although the mortalities incurred in Experimental Series One (Fig. 1 and Table I) posed the formal dilemma of a differential mortality of hermaphrodites at low temperature and of males at high *versus* an experimental induction of males by low temperature and of hermaphrodites by high, nevertheless, the experiments gave strong presumptive evidence of an induction of males by low temperature. The dilemma concerned here usually presupposes a sex-determining mechanism producing about 50% females to 50% males under optimum conditions. This is clearly lacking or inoperative, because under routine laboratory conditions hermaphrodites preponderate over males, no females have been encountered at all, and only hermaphrodites have been encountered in the wild so far. On empirical grounds the expectation is therefore not males and hermaphrodites (or females) in equal numbers but rather a preponderance of hermaphrodites, an expectation not out of line with the mode of reproduction of this species or with the known genetic uniformity of the experimental fish.

The second series of experiments were undertaken to reconfirm the correlation of male incidence with low-temperature rearing, resolve the dilemma of a selective mortality of hermaphrodites *versus* induction of males at low temperature, and delimit somewhat, if existent, that segment of ontogeny within which low temperature can cause a deviation to the male phenotype. These objectives required fewer treatments with more individuals and lower mortalities than in the first series. The three treatments of 50 individuals each were essentially three different temperature regimes (Table IV, A–C). All 150 individuals received light of low daylight intensity. All were reared in fresh water until large enough (see above) to be fed brine shrimp, which were introduced in 40% sea water, after which the fish were gradually changed to 40% sea water, in which they spent the rest of their lives.

The individuals of Group A (controls) were reared throughout at a water temperature of 25 ± 1° C. Those of Group B were under the same conditions through hatching, but the hatchlings were reared for their first five months at 19.5 ± 0.5° C., after which they were maintained at 25 ± 1° C. Those of Group C were reared at 25 ± 1° C. until the eggs were at least in Stage 16 but not beyond Stage 22a (*cf.* Harrington, 1963); then they were reared through eclosion (hatching or cutting out of the chorion) and for five months post-eclosion at 19.5 ± 0.5° C., after which they were maintained at 25 ± 1° C. At the outset of the 19.5 ± 0.5° C. interval of their treatment, 28 of the Group-C individuals that survived to maturity were in Stage 16 (optic vesicles first visible as expansions of the forebrain), 8 in Stage 17 (optocoeles), 3 in Stage 19 (optic cup, lens, and neurocoele), 3 in Stage 20a (optic lobes, neuromeres, pectoral fin-bud anlagen), 3 in Stage 20b (heart pulses without blood circulation), and 1 in Stage 22a (circulation starting through dorsal aorta and vitelline vessels).

Results of Experimental Series Two

The three objectives of this experimental series were achieved with decisive results (Table IV). The zero mortality and absence of males at 25 ± 1° C. (Group A) confirms the correlation of hermaphrodite incidence with high-temperature rearing while showing that the temperature need not be as high as 30° C., as in the first experimental series. This accords with the production of over 95% hermaphrodites to under 5% males when temperatures fluctuated more widely about 25° C., during routine rearing, suggesting that the low percentages of males hatched prior to these experiments resulted from temperatures temporarily below a threshold within 19–24° C. while these fish were traversing a critical segment of organogenesis thermolabile with regard to sex determination and differentiation. The correlation of male incidence with low-temperature rearing was established unequivocally (Group C).

The dilemma of a selective mortality of hermaphrodites *versus* induction of males at low temperature and *vice versa* at high, is resolved, because the mortalities incurred in Groups A–C were zero, and only 8% and 10%, respectively. There can be no doubt that low-temperature rearing caused a deviation to the male phenotype during a critical phase of ontogeny.

The possible extent of this thermolabile phenocritical period of sex determination and differentiation has been contracted at either end. Group B, with 92% survival and all hermaphrodites, not only reinforces the results of Group A, but shows that the thermolabile interval concerned does not extend beyond the end of Stage 31, the last stage before hatching. Group C showed that cold treatment need not begin before Stage 22a (blood circulation just established) to be effective in producing males. This phenocritical period may span a much shorter segment of ontogeny than its above-determined possible maximum extent. Only further experiments of similar design can define it more closely.

Discussion

Uniqueness of the present experiments and results

Of previous experiments investigating possible influences of extrinsic factors on sex determination in fishes, two concerned that rather forced example of environ-

mental influence, overripeness of eggs. In rainbow trout with a delayed fertilization of 21 days, Mršić (1923) reported 55% males, 33% females, and 12% interpreted by him as having ovaries transforming into testes, an interpretation put in doubt by later studies (see below, and Atz, 1964). With moderately delayed fertilizations, he reported small excesses of females. The controls deviated little from the 1:1 sex ratio. Mortalities were more than enough, however, to create the dilemma stated in the introduction to this report: 88% with the 21-day delay, 60–70% with moderate delays, 54% of the controls. Mršić dismissed the dilemma by discounting the mortality as having occurred too early in ontogeny to be the deciding factor, in an argument based on an histological interpretation (see above) of the course of gonad differentiation negated by later studies on rainbow trout (Padoa, 1939) and other salmonids (Ashby, 1952; Robertson, 1953). In brown trout from late-fertilized eggs Huxley (1923) found no significant departure from the 1:1 ratio.

Among broods of Siamese Fighting Fish, Eberhardt (1943) found the sex ratios extremely variable, but under optimum conditions approximating the 1:1 ratio. By crowding during rearing he obtained statistically significant excesses of males, and concluded that poor ("schlechte") space, food, and water conditions favored differentiation in the male direction, *i.e.*, opposite the genetic constitution. Nevertheless, a selective mortality of females cannot be ruled out, because he did not record the mortalities incurred in the experiments. Eberhardt rejected a selective elimination of females not on the basis of the experiments themselves but by inference from the results of rearing 25 other broods on scant food, so as to exaggerate the usual high mortality of the first two weeks of life. Only 4–47 fish survived from these broods of 100–400 hatchlings, and were well fed after the first two weeks. The survivors of 12 broods were 41–50% females; the other broods had somewhat lower percentages of females, but Eberhardt omitted details. The percentages of females in these underfed broods do not adequately support his contention that because in the experiments deaths did not exceed 1% after the first two weeks a selective mortality of females is ruled out. Furthermore, underfeeding and crowding cannot *a priori* be equated with regard to selective mortality, nor can either *a priori* be assumed without influence on sex determination.

A literature too large for more than summary treatment, reviewed in part by Dodd (1960) and Atz (1964), but much of it obsolete, concerns environmental influence on sex determination in the European eel. Grassi (1919) considered temperature, salinity, and nutrition to exert such influence. Forty years later, his student D'Ancona (1960; p. 67) was able to assert merely that his "own experiments suggest the possibility of a phenotypic sex deviation under the influence of experimental factors." Counter to an earlier report (D'Ancona, 1950) that sex ratios ascribed to environmental influences were attributable to a differential migration of the sexes, he named crowding and high temperature as "favoring differentiation toward the male sex," but the evidence is inconclusive and has since been put further in doubt (Sinha and Jones, 1966). It is unfortunate that a species so ill-suited for settling the question of environmental influence on sex determination in fishes became so closely linked with the question historically.

Each *Rivulus marmoratus* individual in the present experiments was reared *ab ovo* in its own container to preclude results attributable to crowding, which would be indecisive as to the proximate extrinsic causal factor. A freemartin-like effect

cannot be dismissed as a possible result of crowding, for not only can sex steroids administered *per os* within a brief period of early ontogeny produce phenotypes of either sex in opposition to the genotypic sex (Yamamoto, 1953–1961), but there is evidence (Egami, 1954) for the uptake by fish in close confinement of estrogenic substances released by other fish (also *cf.* comments of Lindsey, 1962; p. 304). Among the fish crowded by Eberhardt (1943), for instance, rates of growth and sex differentiation varied so much that he resorted to removing the faster-growing ones when these could be sexed externally, each time reconcentrating the sexually indistinguishable ones remaining.

The induction of male gonochorists by incubating the eggs of *R. marmoratus* at low temperature detracts from the proposition that in eels high temperature favors sex differentiation in the male direction, by demonstrating an environmental effect on at least one species of fish the opposite of that on amphibians, in which it is well established (Witschi, 1929, 1957; Piquet, 1930; Uchida, 1937) that high temperature is male-inducing. *R. marmoratus* was chosen for its hermaphroditism and rare male incidence as possibly having a less homeostatic sex-determining mechanism than gonochoristic fishes and thus being a more likely species for testing for environmental influence on sex determination, but the results obtained with *R. marmoratus* raise expectations of analogous results with gonochoristic species of fish. Observations in harmony with our results, but directed to other ends and not excluding a selective mortality of the opposite sex, have in fact been made on two gonochoristic fishes of promise for such experimental testing. In exploring ways of rearing the cyprinodontid fish, *Epiplatys chaperi*, Van Doorn (1962) obtained a higher percentage of males at low temperatures. In experiments on meristic variation, Lindsey (1962) found that rearing conditions of high temperature and crowding produced higher percentages of female sticklebacks, *Gasterosteus aculeatus*.

The activity period of the sex-chromosome genes governing sex determination is equated by Atz (1964; p. 215) with the period of ontogeny in which it is possible with heterotypic hormones to reverse the sex of a gonochoristic fish, *e.g., Oryzias latipes* (Yamamoto, *loc. cit.*). Atz remarks that at present it is problematic whether a similar limited period could be ascribed to hermaphroditic species. Our results bear indirectly on this question. Sex reversal in the sense of transformation from one sexual phenotype (primary gonochorist) to the alternate one (secondary gonochorist) is not concerned here, so that in either hermaphrodite or gonochorist the interval by definition would not extend later than through the sexually indifferent and primary sex-differentiation stages. Both *O. latipes* and *R. marmoratus* hatch sexually undifferentiated and start eating at once as do other cyprinodontids. Making use of these traits by feeding sex steroids to *O. latipes* from the day *after hatching*, Yamamoto caused sex reversals in the sense of producing primary gonochorists of either sex contrary to genotypic sex. In the ontogeny of *R. marmoratus*, the interval within which low-temperature rearing produced males in opposition to the presumed hermaphrodite genotype (see above) begins after onset of blood circulation (possibly long after) but ends *before hatching*. It remains to be determined whether the thermolabile phenocritical period of sex determination in *R. marmoratus* is paralleled, overlapped, or succeeded by a hormonal lability in this respect. In the same context, although the post-hatching hormonal lability of *O. latipes* is not paralleled by a post-hatching thermal lability in *R. marmoratus*, the

peroral administration of sex steroids by Yamamoto in excluding pre-hatching effects, leaves unknown whether the hormonal lability of *O. latipes* begins soon enough to parallel or overlap the pre-hatching thermal lability of *R. marmoratus*. In any case, the much shorter thermolabile interval of sex determination in *R. marmoratus* can be identified with the activity period of the sex-determining genes with as much reason as the interval of 8–10 weeks post-hatching during which Yamamoto fed *O. latipes* the steroids that caused sex reversals. This opens the possibility that teleostean sex determination entails a two-stage sex differentiation, the first stage with thermal lability, the second with hormonal lability.

Exclusion of alernative explanations

The male-inducing effect on *R. marmoratus* of incubation at low temperature emerges as a thermal effect apart from and undisturbed by the structural-functional abnormalities (prolapsed oviduct, pharyngeal hyperplasia, kyphosis) produced by certain (*vide supra*) specific light-temperature-salinity *combinations* of Experimental Series One. The same effect was achieved without these abnormalities, moreover, in Experimental Series Two, which avoided extremes of light intensity, temperature, and salinity, except for low temperature. These abnormalities were confined to dim-light treatments, and are attributable in part at least to hormonal derangements, which further indicates the independence and priority of the thermal effect on sex determination in *R. marmoratus*. Prolapsed oviduct results presumably from either precocious ovulation or abnormal persistence of non-patent oviduct, and in the European Minnow, *Phoxinus phoxinus*, for example, the oviduct becomes patent only within the spawning season, under endocrine control (Bullough, 1939). The pharyngeal hyperplasia and kyphosis in *R. marmoratus* kept at light intensities mostly within the range of civil twilight are reminiscent of the thyroid hyperplasia and kyphosis in the characin, *Astyanax mexicanus*, kept in total darkness, and ascribed to hormonal imbalance normally inhibited by light and involving but not confined to the pituitary-thyroid complex (Rasquin and Rosenbloom, 1954). The dim-light treatments of *R. marmoratus* began right after oviposition, at embryonic stages (Table III) not later than Stage 13c ($\frac{3}{4}$ blastoderm), but eggs of *A. mexicanus* spawned in the light failed to develop in the dark. Rasquin and Rosenbloom placed in darkness specimens kept in the light their first two months of life. Other causes of spinal curvature (Comfort, 1960, 1961) may also have been involved, because not all *A. mexicanus* kept in darkness showed kyphosis, and kyphosis was confined to *R. marmoratus* of only two dim-light treatments, in each of which it fell short of 100% occurrence.

Despite the evident primacy of thermal influence on sex determination and differentiation in *R. marmoratus*, it would constitute the fallacy of misplaced concreteness to conclude that males were produced by low temperature to the complete exclusion of influences from other extrinsic factors. The *principle of complementarity* as extended to biological phenomena (Meyer-Abich, 1956) is especially relevant to environmental influences on the ontogenetic differentiation of aquatic poikilotherms. To identify such influences requires polyfactorial analysis, with combinations of factors controlled as in the present experiments and in such as those of Kinne and Kinne (1962), who observe that not only can one environmental factor

modify the physiological effect of another, but a single factor reaching sufficient intensity to modify the process under study may alter other environmental factors.

The obvious uncontrolled, dependent, extrinsic factor in these experiments on *R. marmoratus* is dissolved oxygen, each egg having been incubated in its own jar of stagnant water. Kinne and Kinne found stagnant (non-aerated) water to have $70 \pm 10\%$ the concentration of dissolved oxygen in aerated (100% air-saturated) water. From their nomograph (Kinne and Kinne, 1962, Fig. 2) can be obtained

TABLE V

Extraparental incubation periods of Rivulus marmoratus *with various combinations of light intensity, salinity, and temperature.* B, bright light; D, dim light; S, sea water; F, fresh water. Same eggs as in Tables I–III; hatched unaided (starred), light-triggered hatching (unencumbered numerals), cut from chorion (parentheses), started at 18° C. and changed to 20° C. (*italicized* numerals), the rest at 20° C. throughout

Numbers of days	Numbers of hatchlings												
	30° C.					18–20° C.							
	Hermaphrodites				Male	Hermaphrodites				Males			
	BS	DS	BF	DF	BF	BS	DS	BF	DF	BS	DS	BF	DF
12	1*												
14	3*	1*	2	2									
15–16	1*	4*		2									
18–19	1*		1	1									
23–24				2									
26–27			3		1								
28								*1**					
30–31				1					1				1
35–36						2+1*		(1)	(1)	1		(1)1	
37–38							1			(1)	(2)1		
39–40							1	(1)1		1	2	1	(1)1
41–42										(1+*1*)	(1)	1	1+1*
43–44										(1)	*1*		
45–46						(1)				(1)	(3)	*2*	*2*
51–56												(*1*)	(*4*)
	14.5	15.0	21.0	19.4	27.0	39.0	38.5	35.3	33.5	40.7	41.0	42.1	44.0

Mean extraparental incubation periods in days.

the approximate 100% air saturation (ml. O_2/L.) for each temperature-salinity combination of our experiments, except those with temperature changed from 18° to 20° C. Although the actual concentrations were less because the water was stagnant, the 100% air saturation values permit an arrangement of the experimental data in order of increasing oxygen concentration (Table VI). In contrast to the decisive thermal influence on sex determination in *R. marmoratus*, not only do these oxygen values fail to uncover evidence of an effect ascribable to oxygen concentration, but in a pilot experiment 10 eggs incubated at 25° C. in 100% air-saturated fresh water yielded 10 hermaphrodites.

Significance of the temperature effect per se

The production of male *R. marmoratus* by low-temperature incubation allows its examination in relation to corresponding rates of embryonic development. The developmental rates of *Cyprinodon macularius* exposed to a diversity of temperature-salinity-oxygen combinations were measured by Kinne and Kinne (1962) as numbers of days from fertilization to certain embryonic stages, especially hatching. These rates increased with increasing oxygen content, and decreased with increasing salinity, the latter effect mediated by changing coefficients of oxygen absorption and saturation in water. Both the retardation and the acceleration were increasingly accentuated by increase in temperature. For comparisons of developmental rates among *R. marmoratus* eggs exposed to different extrinsic factor combinations we must rely on the incomplete data (Table V) of Experimental Series One, because in Experimental Series Two exposure to low temperature was begun at a later and wider range of embryonic stages and the low-temperature embryos were cut from

TABLE VI

The results of Experimental Series One and Two arranged in order of increasing oxygen concentration at 100% air saturation. The actual concentrations were less, because the water was stagnant. See Discussion and Tables I and IV

Approximate temperature	30° C.	20° C.	30° C.	25° C.	20° C.	20° C.
ml. O_2/L. (100% air sat.)	4.3	5.3	5.6	6.0	6.6	6.6
Experimental Series	One	One	One	Two	One	Two
Total eggs	17	31	29	50	26	50
Percentage survival	64.7	67.7	51.7	100.0	53.8	90.0
Percentage male	0.0	48.0	3.4	0.0	30.8	72.0
Percentage hermaphrodite	64.7	19.7	48.3	100.0	23.0	18.0

their chorions far in advance of the normal hatching stage (see above and Table IV, C). In Experimental Series One some eggs hatched unaided, others, with artificial stimulation; the embryos of the rest were cut out but only after some of the same age had hatched, unaided or aided (Table V). Intraparental (pre-treatment) incubations ranged at most from one to 24 hours (Table III and Harrington, 1963, Table I), treatment starting right after oviposition. The incubation periods of Table V might have diverged somewhat more had not many of them been ended arbitrarily, but most of the embryos cut out or from eggs stimulated to hatch would otherwise have perished unidentifiable as to ultimate sex type, as attested by mortalities (Table I) ascribable to extraparental incubations protracted by failure of the hatching mechanism.

The data of Table V, however imperfect, suffice to show a more delayed hatching at high temperature in stagnant fresh water than in stagnant sea water, which is paradoxical with reference to the eggs of *Cyprinodon macularius* (see above and Kinne and Kinne, 1962, Table X). Even if this perhaps resulted from impaired responsiveness of the hatching mechanism, it is no less interesting to find that the single, anomalous male produced at high temperature in our experiments had an extraparental incubation of 27 days in contrast to an average of 17 for the hermaph-

rodites otherwise produced at 30° C. The arbitrary curtailment of the incubations of many of the eggs at low temperature permits only the general comment that there are indications of a possible tendency toward longer incubations among the eggs later found to have yielded males and that the incubations at 20° C. were abnormally long for *R. marmoratus*. At the latitude of the wild-caught founder stock, which is at or near the northernmost extent of the geographic range of this chiefly tropical species, air-temperature daily minima between mid-April and mid-October form a platean at 20° C., the daily means and maxima being much higher, of course. Most of the potential extent of the as yet undefined natural spawning season of *R. marmoratus* is excluded thereby from temperatures of sufficient duration low enough to produce males. Sooner or later, however, some males may be expected to be found in the wild at this latitude hatched from eggs incubated at lower temperatures toward the extremities of the spawning season or perhaps subjected to less obvious, alternative male-inducing conditions like the anomalous, lone male obtained at 30° C. (Tables I–III, V and VI).

The complex effects of temperature *per se* on morphological differentiation and the consequent impossibility of exactly equating developmental stages between embryos incubated at contrasting temperatures hardly needs stating. The imprecision of the classical embryonic "stage" was illustrated by Hayes (1949) with the comment added that hatching itself is not to be regarded as a stage, because it can occur so variably. Nevertheless, with cautionary reservations and for want of anything better, use must still be made of such "stages," sometimes even hatching, as was done by Kinne and Kinne (1962). Hatching as a stage is of normative importance here only in that experiment (Table IV, B) of Experimental Series Two in which low temperature treatment began with hatchlings from eggs incubated at our standard laboratory temperature (25 ± 1° C.). The mean extraparental incubation of the eggs of Table IV, B was 15.3 ± 3.7 days, the total incubation (extra- plus approximate intraparental) was 17.2 ± 3.7 days, and the feeding of each hatchling for one full day at 25 ± 1° C. gave a mean of 19.4 ± 3.8 days before transfer to low temperature. Accumulated laboratory records for 190 other eggs incubated at 25 ± 1° C. yielded a mean extraparental incubation of 17.3 ± 4.5 days and an approximate total incubation of 18.6 ± 4.4 days.

The very phenomenon under consideration, *viz.*, the production of males by extraparental incubation at low temperature, may itself be the result of an uncoupling of embryonic processes (*cf.* Hayes, 1949) by differential effects of low temperature on two or more constituent rates of development, so as to change the order of morphological events critical for sex differentiation. A paradigm for such an effect is the delay by low temperature of medullary development in amphibian gonads that feminizes males, at least temporarily (Uchida, 1937; Witschi, 1957). Although *medulla* as a topographic term has been declared inapplicable to teleostean gonads (D'Ancona, 1952), the bipotential gonocytes are sexualized as ovogonia and spermatogonia, respectively, in heterologous somatic territories within the ovotestes of several hermaphroditic fishes. Nor has uncertainty over the embryogenesis of the heterologous tissues deterred postulations of inductor substances in fishes analogous to the *corticin* and *medullarin* of Witschi, *viz.*, *gynogenine* and *androgenine* by D'Ancona (1949), *gynotermone* and *androtermone* by Yamamoto (1962).

Implications for the interpretation of intersexuality in fishes

Past studies of hermaphroditic fishes have been based at best on histological sections of gonads from economically feasible numbers of fish, sampling as wide a size range as collections provided. In most cases size was the sole criterion of relative age, an unreliable one for fishes, because there may be differential growth rates and mortalities between the sexes, including determinate *versus* indeterminate growth. Interpretations of otherwise adequate samples have been rendered inconclusive or incomplete by uncertainty over the relative ages of the fish coupled with the fact that the effects on growth rate of sex inversion and reversal are unknown. These difficulties are avoided with *R. marmoratus,* which is the first hermaphroditic fish species to have been kept in the laboratory throughout life. The results of the present experiments in conjunction with the daily observation of the fish of Experimental Series One throughout their lives throw light on aspects of fish intersexuality hitherto obscure, because the age and history of each fish were known exactly.

Before applying the results of the present study to these aspects of fish intersexuality, it is pertinent to reassess the extent to which the life span of *R. marmoratus* was encompassed by the experimental and post-experimental observations of Experimental Series One. Several tokens of senility (Comfort, 1960, 1961; Walford and Liu, 1965) appeared among these fish, *e.g.*, clouded cornea, emaciation, exophthalmos, humped back, raising of scales, renal concretions. They were kept alive as long as 1,376 days post-hatching; most of those killed were already *in extremis.* The life span of *R. marmoratus* seems to be of the order of that of another cyprinodont, the poeciliid *Lebistes reticulatus.* Under laboratory conditions, *Lebistes* has a limiting age of 2,000 days, 50% of age-dependent deaths occurring by the end of 800–900 days (Comfort, 1961). Survival both of *Rivulus* and of *Lebistes* to the more advanced ages reached in the laboratory (see Post-treatment Observations) is probably negligible in the wild.

Rivulus marmoratus is the only fish species known to exemplify the ultimate mode of synchronous hermaphroditism, normal self-fertilization. The only other synchronously hermaphroditic fishes known are the serranids, *Serranus cabrilla, S. hepatus, S. scriba* and *S. subligarius,* none of which are claimed to be naturally self-fertilizing (Clark, 1959; Reinboth, 1962; Atz, 1964). The life cycle of *S. subligarius* is incompletely known. The other three species mature and function first as males, the next year first functioning as synchronous hermaphrodites, but it is not known whether all start out as males or some start as hermaphrodites their first year (Reinboth, 1962). On the other hand, *R. marmoratus* is self-fertilizing from outset of functional maturity, even when this is precocious, at higher than usual temperatures. In a sense, therefore, neither these serranids nor *R. marmoratus* are obligate synchronous hermaphrodites throughout life, some or all of the serranid individuals serving as males their first year and some individuals of *R. marmoratus* ending their lives as functional secondary male gonochorists, at least under life-prolonging laboratory conditions. The distinction between synchronous and successional hermaphroditism in fishes thus seems to be one of degree. Of the two forms of successional hermaphroditism, protogyny and protandry, protogyny is the one toward which the synchronous hermaphroditism of *R. marmoratus* leans in tending toward transformation in the male direction, but protogyny is characterized

by temporal succession to the functional male state from the functional female state and not from a fully functional synchronous hermaphroditism, primary and of long duration, as in *R. marmoratus*.

The induction of primary male gonochorists by low-temperature incubation, added to the spontaneous inversion of older hermaphrodites into secondary male gonochorists, are attributes of *R. marmoratus* recalling that primary and secondary males occur also among wrasses (Labridae), *e.g.*, *Coris julis* and *Thallasoma bifasciatum* (Reinboth, 1962). Although secondary male wrasses originate by sex reversal from a primary female condition instead of by sex inversion from synchronous hermaphrodites, perhaps all or some primary male wrasses arise from incubation at low temperature like the primary males of *R. marmoratus*, the no more likely alternative being a homogamety-heterogamety (*e.g.*, XX-XY) switch mechanism yielding protogynous hermaphrodites *versus* primary males, respectively, or *vice versa*. Wrasses have pelagic eggs of short incubation and spawning seasons (Breder and Rosen, 1966) such as to encourage a search for differential latitudinal occurrences of primary males correlated with differential thermal exposures of the drifting eggs. Control of male coloration, however, seems to differ between *Rivulus* and these wrasses. The inference of Zahl (1934), that the caudal ocellus of *Rivulus urophthalmus* is a sex-limited trait suppressed by testicular secretion is strengthened by its presence in all immature *R. marmoratus* also, and even more by its persistence in the hermaphrodites (Figs. 2 and 3), progressive extinction if these transform into secondary males, and usually complete disappearance in primary males (Figs. 2 and 3) at sexual maturity. The striking dichromatism between primary and secondary male wrasses seems to have a more complex hormonal control (Reinboth, 1962; comments of Atz, 1964).

Although *Rivulus marmoratus* exhibits a prolonged synchronous hermaphroditism, its sexuality is more like that of wrasses, *e.g.*, *Coris julis*, than of any other hermaphroditic fishes, with either synchronous or successional hermaphroditism. As far as they have been described, hermaphroditic fishes other than the wrasses and *R. marmoratus* have structurally bisexual gonads alone, although one or the other of the heterologous gonadal territories or dispersed centers may be in a state of persistent abortiveness (rudimentary hermaphroditism), prefunctional latency, or postfunctional involution. The wrasses and *R. marmoratus* also have gonads of bisexual structure. In the wrasses, these function first as ovaries, then, after a short transitional period, as testes, their possessors changing from functional females to functional secondary males. In *R. marmoratus*, they function from the first as ovotestes (Fig. 1, c and d), then after involution of the ovarian component and further evolution of the testicular, as testes alone, their possessors changing from self-fertilizing hermaphrodites for the greater part of their lives to functional secondary male gonochorists for the remainder. Unlike all other hermaphroditic fishes, however, these wrasses and *R. marmoratus* include, besides individuals with bisexual gonads, a minority of primary male gonochorists, with testes of unisexual structure (Fig. 2, e), in contrast to the bisexual structure of the testes of secondary male gonochorists.

The testes of primary males of *R. marmoratus*, and of *Coris julis* (Reinboth, 1962), are like those of the true gonochoristic species making up the majority of fishes, and can easily be told apart from testes of secondary male gonochorists, which

retain the oviduct functional during the female or hermaphroditic phase of the bisexual gonad, and often ovarian residua besides. Testes of true gonochoristic fishes and of the primary males of *R. marmoratus* and *Coris julis* in being of unisexual structure, without oviducal or ovarian vestiges, differ also from the testes of protandrous fishes and of nominal gonochoristic fishes functionally gonochoristic throughout life but with incipient bisexual structure. Testes of the last two categories are so similar that Reinboth (1962, Fig. 27; reprinted as Fig. 2 of Atz, 1964) refers both of them to the same cross-section diagram (Fig. 27, c), which stands in marked contrast to that for the testes of primary males of *Coris julis* (Fig. 27, a). What is labeled *oviduct* in Reinboth's diagrams is of dual origin, the anterior paired lumina derived from the entovarial sulci and nonhomologous with the intratesticular sperm ducts according to Eggert (1933), the unpaired posterior duct arising differently.

In the case of the wrasses, the alternative of primary male *versus* protogynous hermaphrodite might possibly be decided by a genetic shift mechanism instead of by low-temperature incubation, but this is not the case with *R. marmoratus*. In the critical and decisive Experimental Series Two, not only were all individuals of the same clone but they were the end products of selfing through 9–11 uniparental laboratory generations. Since then, further evidence (unpublished) for the homozygosity of these fish has been accumulating from hybridization-*cum*-grafting experiments. All of these lines of evidence of the overwhelming prevalence of hermaphrodites point to the likelihood that the fish of this clone are not only homozygous, but, inferentially, homogametic as well, both hermaphrodites and experimentally produced primary male gonochorists. The fact that both natural and experimentally induced departures from the modal phenotypic expression of the hermaphrodite genotype of *R. marmoratus* are in the male direction, makes it intriguing to find that among amphibians steroid hormones seem capable of causing sex reversal only in the homogametic sex, *i.e.*, to the phenotype of the heterogametic sex (Witschi, 1957). In the fish, *Oryzias latipes*, however, fully functional sex reversals in either direction are produced with sex steroids (Yamamoto, 1953–1961). In *R. marmoratus*, the hermaphroditic constitution is manifestly the epistatic one and the male constitution, the hypostatic. While males result from low-temperature incubation or from sex inversion late in life, females are non-existent. In pilot experiments in which eggs were incubated at a descending series of constant temperatures, 31.2° C. was the highest at which eggs survived, the 10 eggs concerned yielding 10 hermaphrodites.

Breder and Rosen (1966) construe the hermaphroditism of *R. marmoratus* as a mechanism evolved to compensate for the vicissitudes of the unstable coastal mainland and oceanic island environments, allowing reproduction in spite of severe population depletions by violent coastal storms. By the same tokens, if *R. marmoratus* proves to be hermaphroditic throughout its range, which arches island-to-island across the Caribbean from off the Venezuelan coast onto the Florida peninsula, it is easy to see how island-hopping hermaphrodite castaways might found new colonies more readily than males and females of gonochoristic species, with little chance of meeting. Haldane (1957) argues similarly for the colonization of new rivers by flood-transported hermaphroditic or parthenogenetic fish, while noting that if the selfed immigrant ancestor were homogametic the progeny would be more likely to

die out, because of the absence of males. Whatever the long-range prospects of survival for *R. marmoratus*, the graft tests for histocompatibility between wild-caught progenitors and their earliest laboratory descendants make it inescapable that selfing had gone on in the wild for some time. In the long run, perhaps enough males (primary or secondary gonochorists) are produced in the wild to contribute genetic information occasionally from their clone to eggs of another clone, by mating with a hermaphrodite with ovotestes out of phase so as to emit unfertilized eggs. Hermaphrodites do occasionally emit a spate of unfertilized eggs (Harrington, 1963) and we have had apparent success in fertilizing a few of these with sperm from a male of different clone. Attempts to mate males with hermaphrodites have resulted in sexual coaction, but the hermaphrodites, whether previously isolated or not, have emitted eggs in stages of development so advanced as to indicate self-fertilization long before the pairing. In sum, amphimixis between eggs from hermaphrodites of one clone and sperm from primary or secondary male gonochorists of a different clone is in the realm of possibility, but the evidence so far is that it has occurred rarely if at all in the local populations from whence our stock was derived.

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Summary

1. *Rivulus marmoratus* is the only known hermaphroditic fish species naturally self-fertilizing. Tissue grafts between wild-caught fish and their uniparental laboratory descendants give the *autograft reaction*, indicating propagation by selfing in the wild also. Only hermaphrodites have been found in the wild locally, although selfing through more than 10 uniparental laboratory generations yielded a few primary male gonochorists, under 5% in contrast to over 95% that were hermaphrodites. Females seem to be non-existent.

2. Two series of experiments were undertaken to identify a possible environmental factor able to cause a deviation to the male phenotype during sex differentiation, on the working hypothesis that low male incidence in clones composed otherwise of hermaphrodites indicated a lability in the sex-determining mechanism through which the genotype normally produces the hermaphrodite phenotype.

3. Individuals of two clones, each in its own jar throughout life, were exposed to the eight combinations of bright or dim light, sea water or fresh water, high or low temperature (Experimental Series One). Exposure was from not later than the $\frac{3}{4}$ blastoderm stage until sexual maturity at high temperature or five months post-hatching at low.

4. Over seven times the number of males previously encountered were obtained, all but one from low-temperature treatments. Male production was correlated with

low-temperature rearing despite alternative light intensities and salinities and structural-functional abnormalities (*prolapsed oviduct, pharyngeal hyperplasia, kyphosis*) peculiar to different dim-light, salinity-temperature combinations, and partly attributable to hormonal derangements. Mortalities were high enough to present the formal dilemma of a differential male induction *versus* hermaphrodite mortality at low temperature and *vice versa* at high, but this dilemma was resolved by Experimental Series Two.

5. The Experimental Series One fish were monitored daily up to 1,376 days post-hatching, by which time almost 60% of the hermaphrodites had changed to functional secondary male gonochorists, the rest dying or killed as hermaphrodites, some each year. Primary males remain unchanged except for senile degeneration. Secondary males arise mostly late in laboratory-prolonged life, by involution of the ovarian component of the ovotestes with further evolution of the testicular component, the caudal ocellus fading or vanishing as they become orange like the primary males.

6. In Experimental Series Two, mortalities were low and the structural-functional abnormalities were absent. All individuals were kept at the same intermediate salinity and light intensity: Group A, at moderate temperature throughout to maturity; Group B, at the same temperature through hatching, at low temperature the first five months post-hatching, thereafter at the moderate temperature; Group C, at the moderate temperature up to stages from optic vesicle formation to outset of blood circulation, then at low temperature through eclosion and for five months post-eclosion. Group-C embryos being cut from their chorions to minimize deaths from hatching failure.

7. The Group-A eggs yielded 100% hermaphrodites, the Group-B eggs, 92% hermaphrodites and 8% deaths, the Group-C eggs, 72% males, 18% hermaphrodites, and 10% deaths. Exposure to low temperature from as late as outset of blood circulation produced males.

8. The uniqueness of the present experiments and results, exclusion of alternative explanations, significance of the temperature effect *per se*, and the implications of these findings for the interpretation of intersexuality in fishes are discussed at length.

LITERATURE CITED

Ashby, K. R., 1952. Sviluppo del sistema riproduttivo di *Salmo trutta* L., in condizioni normali e sotto l'influenza di ormoni steroidi. *Riv. Biol.*, n. s. **44**: 3–18.

Atz, J. W., 1964. Intersexuality in fishes. *In*: Intersexuality in Vertebrates Including Man (ed. by C. N. Armstrong and A. J. Marshall), Academic Press, London and New York.

Breder, C. M., Jr., and D. E. Rosen, 1966. Modes of Reproduction in Fishes. The American Museum of Natural History, Natural History Press, Garden City, New York.

Bullough, W. S., 1939. A study of the reproductive cycle of the minnow in relation to the environment. *Proc. Zool. Soc. London, Ser. A,* **109**: 79–102.

Clark, E., 1959. Functional hermaphroditism and self-fertilization in a serranid fish. *Science,* **129**: (3343): 215–216.

Comfort, A., 1960. The effect of age on growth resumption in fish (*Lebistes*) checked by food restriction. *Gerontologia,* **4**: 177–186.

Comfort, A., 1961. The longevity and mortality of a fish (*Lebistes reticulatus* Peters) in captivity. *Gerontologia,* **5**: 209–222.

D'Ancona, U., 1949. Ermafroditismo ed intersessualità nei Teleostei. *Experientia,* **5**: 381–389.

D'Ancona, U., 1950. Détermination et différenciation du sexe chez les poissons. *Arch. Anat. Micros. et Morph. Exp.*, **39**: 274–292.

D'Ancona, U., 1952. Territorial sexualization in the gonads of teleosteans. *Anat. Rec.*, **114**: 666–667.

D'Ancona, U., 1960. The life-history of the Atlantic eel. *In*: Hormones in Fish (ed. by I. Chester Jones), *Symposia Zool. Soc. London*, No. 1.

Dodd, J. M., 1960. Genetic and environmental aspects of sex determination in cold-blooded vertebrates. *Mem. Soc. Endocrinol., No.* **7**: 17–44.

Eberhardt, K., 1943. Geschlechtsbestimmung und -differenzierung bei *Betta splendens* Regan. *Zeitschr. für indukt. Abstamm. und Vererbungslehre*, **81**: 363–373.

Egami, N., 1954. Influence of temperature on the appearance of male characters in females of the fish, *Oryzias latipes*, following treatment with methlydihydrosterone. *J. Fac. Sci., Univ. Tokyo*, Sec. IV, **7** (2): 281–298.

Eggert, B., 1933. Die Intersexualität bei Knochenfischen. *Zeitschr. wiss. Zool.*, **144**: 402–420.

Garman, S., 1895. The cyprinodonts. *Mem. Mus. Comp. Zool.*, **19** (1): 1–179.

Gordon, M., 1957. Physiological genetics of fishes. *In*: The Physiology of Fishes (ed. by M. E. Brown), Vol. 2, Academic Press, New York.

Grassi, B., 1919. Nuove ricerche sulla storia naturale dell'Anguilla. *Mem. R. Com. Talass. Ital.*, **67**: 1–141.

Haldane, J. B. S., 1957. Sex determination in Metazoa. *Proc. Zool. Soc. Calcutta*, Mookerjee Memorial Volume **1957**: 13–28.

Harrington, R. W., Jr., 1959. Delayed hatching in stranded eggs of marsh killifish, *Fundulus confluentus*. *Ecology*, **40** (3): 430–437.

Harrington, R. W., Jr., 1961. Oviparous hermaphroditic fish with internal self-fertilization. *Science*, **134** (3492): 1749–1750.

Harrington, R. W., Jr., 1963. Twenty-four-hour rhythms of internal self-fertilization and of oviposition by hermaphrodites of *Rivulus marmoratus*. *Physiol. Zoöl.*, **36** (4): 325–341.

Harrington, R. W., Jr., and L. R. Rivas, 1958. The discovery in Florida of the cyprinodont fish, *Rivulus marmoratus*, with a redescription and ecological notes. *Copeia*, **1958** (2): 125–130.

Hayes, F. R., 1949. The growth, general chemistry, and temperature relations of salmonid eggs. *Quart. Rev. Biol.*, **24**: 281–308.

Hoedeman, J. J., 1958. Rivulid fishes of the Antilles. *Studia Fauna Curaçao*, **8**: 112–126.

Huxley, J. S., 1923. Late fertilization and sex-ratio in trout. *Science*, **58** (1502): 291–292.

Kallman, K. D., and R. W. Harrington, Jr., 1964. Evidence for the existence of homozygous clones in the self-fertilizing hermaphroditic teleost *Rivulus marmoratus* (Poey). *Biol. Bull.*, **126**: 101–114.

Kinne, O., and E. M. Kinne, 1962. Rates of development of embryos of a cyprinodont fish exposed to different temperature-salinity-oxygen combinations. *Canad. J. Zool.*, **40**: 231–253.

Lindsey, C. C., 1962. Experimental study of meristic variation in a population of threespine sticklebacks, *Gasterosteus aculeatus*. *Canad. J. Zool.*, **40**: 271–312.

Meyer-Abich, A., 1956. The principle of complementarity in biology. *Acta Biotheoretica, Ser. A*, **11**: 57–74.

Mršić, W., 1923. Die Spätbefruchtung und deren Einfluss auf Entwicklung und Geschlechtsbildung, experimentell nachgeprüft an der Regenbogenforelle. *Arch. f. Entw.*, **98**: 129–209.

Nielsen, E. T., 1961. Twilight and the "crep" unit. *Nature*, **190** (4779): 878–879.

Nielsen, E. T., 1963. Illumination at twilight. *Oikos*, **14** (1): 9–21.

Nogusa, S., 1960. A comparative study of the chromosomes in fishes with particular consideration on taxonomy and evolution. *Mem. Hyogo Univ. Agric.* (Biol.), **3** (1): 1–60.

Padoa, E., 1939. Observations ultérieures sur la différenciation du sexe, normale et modifiée par l'administration d'hormone folliculaire, chez la Truite iridée (*Salmo irideus*). *Bio-Morphosis*, **1**: 337–354.

Pflüger, E., 1882. Versüche der Befruchtung überreifer Eier. *Pflüger's Archiv für gesamte Physiol. des Menschen und der Tiere*, **29**: 76–77.

PIQUET, J., 1930. Détermination du sexe chez les batraciens en fonction de la température. *Rev. Suisse Zool.*, **37**: 173–281.

RASQUIN, P., AND L. ROSENBLOOM, 1954. Endocrine imbalance and tissue hyperplasia in teleosts maintained in darkness. *Bull. Amer. Mus. Nat. Hist.*, **104** (4): 365–425.

REINBOTH, R., 1962. Morphologische und functionelle Zweigeschlechtlichkeit bei marinen Teleostieren (Serranidae, Sparidae, Centracanthidae, Labridae). *Zool. Jahrb. Abt. für Zool. und Physiol. der Tiere*, **69**: 405–480.

RIVAS, L. R., 1945. The discovery and redescription of the types of *Rivulus marmoratus* Poey, a cyprinodont fish from Cuba. *J. Wash. Acad. Sci.*, **35** (3): 95–97.

ROBERTSON, J. G., 1953. Sex differentiation in the Pacific salmon *Oncorhynchus keta* (Walbaum). *Canad. J. Zool.*, **31** (2): 73–79.

ROTHSCHILD, LORD, 1958. Fertilization in fish and lampreys. *Biol Rev.*, **33**: 372–392.

SINHA, V. R. P., AND J. W. JONES, 1966. On the sex and distribution of the freshwater eel (*Anguilla anguilla*). *J. Zool., Lond.*, **150** (3): 371–385.

SINNOTT, E. W., AND L. C. DUNN, 1939. Principles of Genetics. 3rd ed., McGraw-Hill Book Co., New York and London.

SPURWAY, H., 1957. Hermaphroditism with self-fertilization and the monthly extrusion of unfertilized eggs in the viviparous fish *Lebistes reticulatus*. *Nature*, **180** (4597): 1248–1251.

UCHIDA, T., 1937. Studies on the sexuality of Amphibia. III. Sex-transformation in *Hynobius retardatus* by the function of high temperature. *J. Fac. Sci. Hokkaido Imper. Univ., Ser. VI*, **6**: 59–70.

VAN DOORN, W. A., 1962. Geschlachtsbeinvloeding door temperatur. *Het Aquarium*, **32** (9): 208–209.

WALFORD, R. L., AND R. K. LIU, 1965. Husbandry, life span, and growth rate of the annual fish, *Cynolebias adloffi* E. Ahl. *Exp. Geront.*, **1**: 161–171.

WHITE, M. J. D., 1954. Animal Cytology and Evolution. 2nd ed., Cambridge University Press, Cambridge, England.

WITSCHI, E., 1929. Studies on sex differentiation and sex determination in amphibians. II. Sex reversal in female tadpoles of *R. sylvatica* following the application of high temperature. *J. Exp. Zool.*, **52**: 267–291.

WITSCHI, E., 1957. The inductor theory of sex differentiation. *J. Fac. Sci. Hokkaido Univ., Ser. VI*, **13** (Professor T. Uchida Jubilee Volume): 428–439.

YAMAMOTO, T., 1953. Artificially induced sex-reversal in genotypic males of the Medaka (*Oryzias latipes*). *J. Exp. Zool.*, **123**: 571–594.

YAMAMOTO, T., 1958. Artificial induction of functional sex-reversal in genotypic females of the Medaka (*Oryzias latipes*). *J. Exp. Zool.*, **137**: 227–260.

YAMAMOTO, T., 1959. The effects of estrone dosage level upon the percentage of sex-reversals in genotypic male (XY) of the Medaka (*Oryzias latipes*). *J. Exp. Zool.*, **141**: 133–154.

YAMAMOTO, T., 1961. Progenies of sex-reversal females mated with sex-reversal males in the Medaka, *Oryzias latipes*. *J. Exp. Zool.*, **146**: 163–179.

YAMAMOTO, T., 1962. Hormonic factors affecting gonadal sex differentiation in fish. *Gen. Comp. Endocrin., Suppl.*, **1**: 341–345.

ZAHL, P. A., 1934. The hormonic inhibition of a somatic character in the cyprinodont fish, *Rivulus urophthalmus*. *Amer. Nat.*, **68**: 475–477.